lymph node disease than in other diseases; however, this difference was not significant (44% vs 27%, 89% vs 50%), respectively. CR was achieved in two cases of lymph node disease and in ten cases of other disease sites. In both sensitive and refractory/resistant cases, response rate and non-PD rate tended to be higher in lymph node disease. The relationship between chemotherapy response and recurrent sites in 12 multiple recurrent cases is shown in Table 3. In eight of 12 cases, similar chemotherapy responses were obtained despite differing disease sites. In four of 12 cases (case 8, 9, 10, 11), chemotherapy responses were different among recurrent sites. In two cases (10 and 11) chemotherapy responses for lymph node disease were stable, however, responses for other recurrent sites were PD.

Discussion

Recurrence of EOC are almost always fatal. For recurrent EOC, therapeutic options consist of surgery, chemotherapy, and radiotherapy. The NCCN guidelines recommend surgical treatment for clinically focal recurrence after a disease-free interval > 6 months. Recently, retrospective studies have shown that secondary cytoreductive surgery for isolated nodal recurrence was effective [9-12]. However, there have been no high-quality reports which compared salvage chemotherapy with surgery for focal recurrence after a disease-free interval > 6 months. Before 1990, lymphadenectomy was often performed at a second-look operation after chemotherapy; positive nodes were found just as frequently at second-look operations as in patients undergoing lymphadenectomy at primary surgery [17-19]. Recently, Morice *et al.* examined the rates of nodal involvement in 205 EOC patients and reported that the rates of nodal involvement in patients who underwent lymphadenectomy prior to or after chemotherapy were not statistically different [13]. These findings may indicate that chemotherapy may have little effect against the retroperitoneal lymph nodes metastases. In contrast, Banchard *et al.* reported that a good response rate could be obtained for lymph node metastasis (11 CR out of 20 treated patients) [10]. In this study, response rate and non-PD rate for lymph node diseases were 100% and 100% for sensitive cases, and 0% and 80% for refractory/resistant cases, and the chemotherapy effect for lymph node disease tended to be better than that for other recurrent sites.

In contrast, response rate and non-PD rate for liver diseases were 33% and 33% for sensitive cases, and 0% and 29% for refractory/resistant cases. Kusumoto *et al.* examined the chemosensitivity of 16 pairs on samples obtained simultaneously from primary and metastatic lesions of clinical gastric cancer by in vitro chemosensitivity test (succinate dehydrogenase inhibition test) and reported that the lymph nodes were more chemosensitive to carboquone, doxorubicin, mitomycin C, cisplatin, aclacinomycin A and 5-FU, while the liver was less sensitive than the primary lesions to carboquone, doxorubicin, mitomycin C, cisplatin, aclacinomycin A and 5-FU [20]. These findings are concordant with the findings of this study.

The effect of chemotherapy on survival for isolated lymph node relapse was thought to be essential to conclude the chemotherapy effect. However, there were only three cases who had isolated lymph node relapse in this study. The remaining six cases with lymph node relapse were accompanied by other recurrent diseases. Isolated lymph node relapse of EOC is reported to be a rare event and its prevalence has been reported to be about 5% [5, 9-12, 21].

In conclusion, response rate and non-PD rate for lymph node disease tended to be relatively high. Further study analyzing survival will be required to conclude the chemotherapy effect.

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MicroRNA-34b functions as a potential tumor suppressor in endometrial serous adenocarcinoma

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Endometrial serous adenocarcinoma (ESC) is aggressive and carries a poor prognosis. p53 is frequently mutated in ESC. microRNAs (miRNAs) are a direct p53 target and have been implicated in cancer cell behavior. In this study, we compared miRNA expression levels in ESC with the levels in endometrial endometrioid adenocarcinoma (EEC) and normal endometria. Six miRNAs were identified as having aberrant down-regulation specific to ESC with miR-34b being most pronounced. miR-34b was found to have promoter hypermethylation, which when reversed, restored miR-34b expression in the cell lines treated with 5-aza-2′ deoxycytidine (DAC). Ectopic expression of miR-34b in turn inhibited cell growth, migration and most notably invasion. Our findings suggest a relationship among p53 mutation, miR-34b promoter methylation and tumor cell behavior. These effects are likely mediated by the downstream target of miR-34b, the proto-oncogene mesenchymal-epithelial transition factor (MET), a known prognostic factor in endometrial carcinomas. The expression of MET was reduced following the restoration of miR-34b in cell lines. In summary, our data suggest that miR-34b plays a role in the molecular pathogenesis of endometrial cancer.

Introduction

Endometrial serous adenocarcinoma (ESC) accounts for 10% of all endometrial carcinomas. In contrast to the more common Type I endometrial carcinomas, this tumor often presents at an advanced stage with deep myometrial invasion and a high incidence of lymph node involvement. The average age of onset is older than for Type I endometrial carcinoma.[1] The recurrence rate for ESC is high and the 5-year survival rate ranges from 15 to 51%.[2] The prognosis of ESC is, at best, equivalent to that of Grade III endometrial endometrioid adenocarcinoma (EEC) confined to the uterus.[3] The most prominent genetic alteration in ESC, demonstrated in 90% of tumors, is p53 mutation,[4] which frequently manifests as an accumulation of defective p53 protein.[5,6] Although p53 mutation is a common genetic alteration in a variety of tumor types, its role in tumorigenesis, particularly in gynecologic cancers, has not been completely elucidated. Furthermore, conflict exists regarding the utilization of p53 alterations as a prognostic factor. It is plausible that cell context-specific differences in pathways downstream of p53 may also play a role.

Direct targets of p53 include DNA sequences coding for microRNAs (miRNAs). miRNAs are single-stranded, noncoding RNAs of 18–24 nucleotides which have recently been shown to regulate protein expression. miRNAs bind to specific mRNAs, thereby blocking translation and increasing degradation.[7] Several of these mRNA targets code for proteins with oncogene and tumor suppressor functions;[8,9] therefore, by affecting the translation of these genes, miRNAs may play a key role in cellular transformation[10,11] and tumor metastasis.[12]

Members of the miR-34 family (miR-34a, miR-34b and miR-34c) are direct miRNA targets of p53 and represent potential tumor suppressors.[13–18] Expression of these miRNAs appears to be epigenetically regulated. DNA methylation of miR-34b/c has been found in colorectal cancer as well as in melanoma, in which methylation of CpG islands correlates with decreased expression and increased metastatic potential.[19,20] This effect may be mediated by the MET proto-oncogene, which has been identified as a putative target gene of miR-34a.[14]

MET encodes the hepatocyte growth factor receptor, a tyrosine kinase that is associated with invasive ability, cell growth, angiogenesis and scattering.[21] Numerous studies have shown that invasive growth is attenuated by the inhibition of MET expression, indicating a close relationship between MET and invasive properties.[22,23] It is unclear, however, if MET plays a role in ESC.

Key words: microRNA, miR-34b, MET, endometrial cancer, serous
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To investigate whether a relationship existed between miRNA and tumor behavior, we obtained miRNA profiles of endometrial carcinomas using an miRNA microarray. We then sought to identify specific miRNAs, and target mRNAs associated with invasiveness and p53 mutation in ESC. Finally, we investigated how these miRNAs affected the function of endometrial cancer cells.

Material and Methods

Cell lines

Four human endometrial cancer cell lines (Ishikawa, RL95-2, SPAC-1-L and USPC-1) were examined in this study. Ishikawa cells were provided by Dr. Nishida from the Department of Obstetrics and Gynecology, Institute of Clinical Medicine, University of Tsukuba (Ibaraki, Japan). RL95-2 cells were obtained from the American Type Culture Collection (Rockville, MD). Established human endometrial serous carcinoma cell lines were provided by the laboratory of Dr. Hirai (SPAC-1-L), Department of Gynecology, Cancer Institute Hospital (Tokyo, Japan)[24] and Dr. Santin (USPC-1), Department of Obstetrics and Gynecology, Division of Gynecologic Oncology at the Yale University School of Medicine (New Haven, CT).[25] All cell lines were cultured in the appropriate medium and passed at confluence on 10 cm^2 dishes (Becton Dickinson and Co., Lincoln Park, NJ). The dishes were cultured in a 37°C incubator supplied with humidified 5% CO_2 and 95% air. The medium was changed twice a week.

Cells were incubated in growth medium with or without the DNA demethylating agent, 1 μM 5-aza-2′ deoxycytidine (DAC; Sigma-Aldrich, St. Louis, MO), for 72 hr, replacing the drug and medium every 24 hr. For histone deacetylase inhibition, 0.5 μM trichostatin A (TSA; Sigma-Aldrich) was added for the final 16 hr.

Tissue samples

After obtaining informed consent, 21 serous adenocarcinoma tissues, 20 endometrioid adenocarcinoma tissues, and 7 normal endometrial tissues (four proliferative phases and three secretory phases) were retrieved from the surgical pathology files at Tohoku University Hospital (Sendai, Japan). The research protocol was approved by the Ethics Committee at Tohoku University Graduate School of Medicine (Sendai, Japan). All surgical specimens were collected between January 2001 and December 2006 at Tohoku University Hospital (Sendai, Japan). Only patients diagnosed with a pure adenocarcinoma without other histological elements were included. The clinical data and patient characteristics are shown in Supporting Information, Table S1. We also obtained control normal endometrial tissue samples from hysterectomy specimens obtained from patients who underwent surgery for benign conditions. No patients had received preoperative radiotherapy or chemotherapy. The lesions were classified using World Health Organization criteria and were staged according to the International Federation of Gynecology and Obstetrics system.[26,27] The specimens were processed in 10% formalin, fixed for 24–48 hr, paraffin embedded, and thin-sectioned (3 μm). Frozen archival specimens were embedded immediately upon collection in optimal cutting temperature (OCT) compound (Sakura Finetechnical, Tokyo, Japan) and stored at −80°C for further use. Only sections containing a minimum of 90% carcinoma by examination with hematoxylin–eosin staining were used for total RNA and DNA preparation. Total RNA, including miRNA, was extracted using QIAzol Lysis reagent (Qiagen, Valencia, CA) and the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen).

Immunohistochemistry

Immunohistochemical analysis was performed with the streptavidin–biotin amplification method using a Histofine kit (Nichirei, Tokyo, Japan). A monoclonal antibody for p53 (B20.1) and a polyclonal antibody for MET (SP260) were purchased from Biomeda (Foster City, CA) and Santa Cruz Biotechnology, respectively. For immunostaining, the slides were heated in an autoclave at 121°C for 15 min for p53, and in a microwave for 20 min for MET in 0.01 M citric acid buffer following deparaffinization for antigen retrieval. The dilutions of primary antibodies for p53 and MET were 1:50 and 1:100, respectively. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine solution and counterstained with hematoxylin. Tissue sections of colon cancer and breast cancer were used as positive controls for p53 and MET, respectively. For p53 expression, tumor cells were considered positive when more than 10% of the tumor cells showed nuclear staining. For MET expression, the distribution and intensity were scored according to methods which have been described previously.[28] The percentage of positive cells was classified as 0 (none), 1 (<1%), 2 (2–10%), 3 (11–33%), 4 (34–66%) and 5 (>67%). The immunointensity was classified as 0 (negative), 1 (very weak), 2 (weak), 3 (moderate), 4 (strong) and 5 (very strong). The total score of cell was obtained by adding the immunostaining score and the immunointensity score (range, 0–10). Scores from 2 to 10 were regarded as positive, whereas scores from 0 to 1 were regarded as negative. The immunohistochemical expression was independently reviewed by two of the authors (E. H. and J. A).

miRNA microarray analysis

RNA purity and concentration were confirmed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). miRNA microarrays were manufactured by Agilent Technologies and contained 20–40 features targeting each of 470 human miRNAs. Labeling and hybridization of total RNA samples (100 ng) were performed according to the manufacturer's protocol. The arrays were scanned with an Agilent microarray scanner (Agilent Technologies) using high dynamic range settings as specified by the manufacturer.

Microarray results were extracted using Agilent Feature Extraction software Ver. 9.5.3.1 (Agilent Technologies) and analyzed using Gene Spring GX 7.3.1 software (Agilent Technologies) to obtain gene expression ratios. Microarray data have been submitted to the GEO database (GSE25405).

Quantitative real-time reverse transcription polymerase chain reaction

Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis was performed using Taqman MicroRNA Assays (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. Five nanograms of total RNA was used for the synthesis of first-strand cDNA using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. Real-time PCR analyses were performed on an ABI7500 thermalcycler (Applied Biosystems), using TaqMan probes hsa-mir-34b and RNU6B. The fold-change for miRNA, relative to RNU6B, was calculated using the $2^{-\Delta\Delta Ct}$ method.[29] Three independent RT-PCR reactions were performed.

Western blot analysis

Cell protein was extracted using M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL) with Halt Protease Inhibitor Cocktail (Pierce Biotechnology). Ten micrograms of protein (whole cell extracts) was subjected to Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) (10% acrylamide gel). Following SDS-PAGE, proteins were transferred onto Hybond P polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, UK). The membrane was then washed with tris buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) and blocked in TBS-T containing 5% skim milk. The membrane was probed with primary antibodies for MET (Cell Signaling Tech, Danvers, MA) and β-actin (Sigma–Aldrich) for 1 hr at room temperature. Primary antibodies were diluted as follows: MET 1/1,000 and β-actin 1/10,000. After incubation with antimouse IgG horseradish peroxidase (GE Healthcare) for 1 hr at room temperature, antibody–protein complexes on the blots were detected using ECL-plus Western blotting detection reagents (GE Healthcare). The protein bands were visualized with the LAS-1000 image analyzer (Fuji Photo Film, Tokyo, Japan).

Methylation analysis

One microgram of genomic DNA was treated with sodium bisulfite using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. Bisulfite colony sequence analysis was carried out in endometrial cancer cell lines and bisulfite direct sequence analysis in normal and tumor specimens. The primer sequences were designed using Meth Primer (http://www.urogene.org/methprimer/). Hot-start PCR was performed at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 1 min. The correctly sized band was isolated and its DNA was extracted using the QIAquick Gel Extraction Kit (Qiagen). Amplified bisulfite-sequencing PCR products were cloned into the T-easy vector (Promega, Madison), and 10 clones from each sample were sequenced using an ABI3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

PCR and p53 gene mutation analysis

TP53 exons 5–8 were amplified using primers which are described elsewhere.[30] Amplification reactions (20 μL) contained 10 ng of DNA and 3.2 pmol of each primer according to the manufacturer's instructions. The amplifications were performed with a DNA Engine Tetrad, PTC-225 thermal cycler (Biorad, Hercules, CA). PCR products were purified using the NucleoFast 96 PCR plates from Macherey-Nagel using the manufacturer's protocol. Purified PCR products were sequenced using the ABI Prism BigDye terminator v3.1 chemistry (Applied Biosystems) and an ABI 3100 genetic analyzer (Applied Biosystems). The results were analyzed using SeqScape v1.0 (Applied Biosystems). Sequencing reactions were performed in both the forward and reverse directions. All sequences were manually examined. All exons with a mutation were reamplified and resequenced in two directions.

Transfection of precursor miRNA

SPAC-1-L cells (1×10^5) and USPC-1 cells (1×10^5) were transfected with 100 pmol of Pre-miR miRNA Precursor Molecules (Applied Biosystems) or Pre-miR miRNA Molecules Negative Control 1 or 2 (Applied Biosystems) using LipofectamineRNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For the Western blot analysis and biological assays, cells were used 72 hr after transfection.

Cell proliferation assay, apoptosis assays, migration assay and invasion assay

Cell number was evaluated indirectly using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. The apoptotic status of cells was evaluated using a Caspase-3/7 Glo Assay (Promega) according to the manufacturer's instructions. Fluorescence was obtained with a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA). Detection of apoptosis by flow cytometry was performed using the Annexin V-FITC Apoptosis Detection Kit (MBL, Nagoya, Japan). The staining was performed according to the manufacturer's instructions. Ten thousand cells per sample were analyzed using a BD FACSCanto^{TM}II flow cytometer (BD Biosciences). For transwell migration assays, 5×10^4 cells were plated in the top chamber with a noncoated membrane (24-well insert; pore size, 8 μm; BD Biosciences, Two Oak Park, MA). For invasion assays, 5×10^4 cells were plated in the top chamber with a Matrigel-coated membrane (24-well insert; pore size, 8 μm; BD Biosciences). In both assays, cells were plated in serum-free medium containing 1% bovine serum albumin (BSA), and medium supplemented with serum was used as a

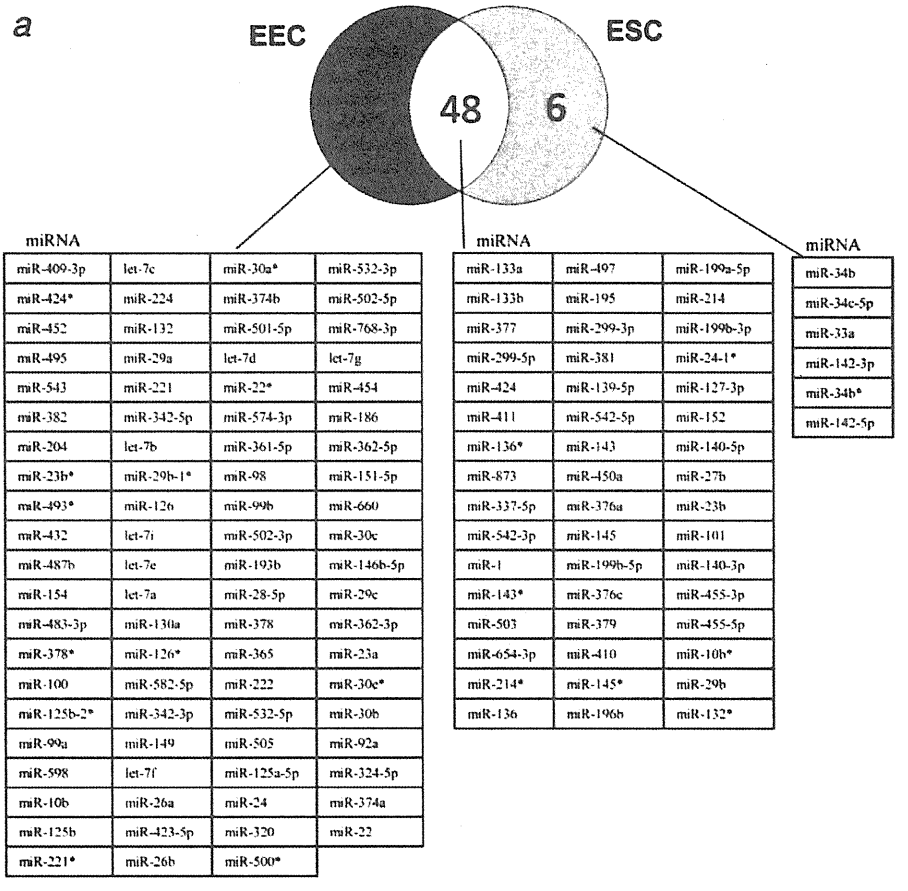

Figure 1. (*a*) miRNAs that exhibited a statistically significant decrease relative to normal endometria. Six genes (pink) were specifically down-regulated in ESC. (*b*) Quantitative real-time RT-PCR of miR-34b microarray data. Expression of miR-34b was significantly down-regulated in ESC. Columns, means of three replicates. Normal, normal endometrial tissues. $^{**}p < 0.01$; NS, nonsignificant. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

chemoattractant in the lower chamber. The cells were incubated for 6 hr at 37°C for the migration assay and 24 hr at 37°C for the invasion assay. Cells which did not migrate or invade through the pores were removed by a cotton swab, and those on the lower surface were subsequently fixed with methanol and stained with toluidine blue O. The migration or invasive ability was evaluated as the total number of cells on the lower surface of the membrane as determined by microscopy. All functional analyses were performed in duplicate and repeated independently three times.

Statistical analysis

Raw microarray data were normalized and analyzed using Gene Spring GX 7.3.1 software (Agilent Technologies). Expression data were median centered. Statistical analysis was performed using SAS software version 5.0 (Statview, Cary, NC). miRNAs that had a greater than twofold change were considered to have significant differential expression compared to normal endometria. The Mann–Whitney *U* test was performed to identify miRNAs that demonstrated statistically significant differential expression, and to evaluate differences between miRNA expression and immunohistochemical expression. Results were expressed as means ± SD and analyzed by one-way analysis of variance (ANOVA) and the Bonferroni test for functional analyses. $p < 0.05$ was considered to be statistically significant.

Results

Analysis and comparison of miR-34b expression in ESC tissues, EEC tissues and normal endometrial tissues

miRNA microarray analysis was used to identify miRNAs that were differentially expressed among ESC, EEC, and normal endometria. We focused on down-regulated miRNAs as many are regulated by P53. Figure 1*a* shows the miRNAs exhibiting statistically significant down-regulation in ESC and EEC compared to normal endometrial tissues. A total of 54 miRNAs were down-regulated in ESC, and 131 in EEC. Forty-eight miRNAs were commonly down-regulated in ESC and EEC. Notably, six miRNAs were specifically down-regulated in ESC. Of these six miRNAs, miR-34b displayed the greatest degree of down-regulation (40.2-fold; Table 1). Down-regulation of miR-34b was validated by quantitative RT-PCR which confirmed significant down-regulation in ESC (35.8-fold, $p = 0.002$), and no significant down-regulation in EEC (2.9-fold, NS; Fig. 1*b*).

We next investigated whether a correlation existed between miR-34b expression in ESC and clinicopathological features (clinical stage, myometrial invasion, lymph node metastasis and degrees of vascular invasion) and found no statistically significant associations (data not shown). p53 immunoreactivity was detected in the nuclei of 18 out of 21 (85.7%) ESC cases (Supporting Information, Table S1 and Fig. S1), while all normal endometrial tissues were negative. Lower expression of miR-34b tended to be inversely correlated with p53 immunohistochemical overexpression; however, this finding was not statistically significant (data not shown).

Analysis of miR-34b CpG island DNA methylation in endometrial cancer cell lines and primary ESC

To determine whether miR-34b down-regulation was explained by DNA methylation, we performed bisulfite sequencing of endometrial cancer cell line genomic DNA. This analysis revealed that the CpG sites in this region were extensively methylated in RL95-2 and SPAC-1-L cells, whereas moderate methylation was seen in USPC-1 cells. In contrast, only a low level of methylation was seen in Ishikawa cells (Fig. 2*a*). The average methylation levels in Ishikawa, RL95-2, SPAC-1-L and USPC-1 cells were 10, 96, 76 and 25%, respectively (Table 2).

Table 1. Specially down-regulation expression miRNAs in ESC *versus* normal endometrial tissue

miRNA	Fold change	*p* value
miR-34b	40.2	0.014
miR-34c-5p	**6.17**	**0.014**
miR-33a	6.1	0.002
miR-142-3p	**3.18**	**0.005**
miR-34b*	2.97	0.016
miR-142-5p	**2.69**	**0.01**

Significant *p* value ($p < 0.05$).

We next analyzed the miR-34b methylation pattern in tumor specimens from ESC patients. Bisulfite sequencing revealed that miR-34b was methylated in 37% of the ESC specimens and in 60% of EEC. In contrast, only a low level of methylation (6%) was detected in normal endometrial samples, indicating that methylation of the miR-34b region is a tumor-specific phenomenon (Fig. 2*a*).

We next analyzed four endometrial cancer cell lines (Ishikawa, RL95-2, SPAC-1-L and USPC-1), which were treated with or without DAC and TSA. We found that miR-34b was markedly up-regulated or re-expressed by DAC in RL95-2, SPAC-1-L and USPC-1. Up-regulation did not occur in Ishikawa cells. DAC treatment produced increases in miR-34b expression of the following magnitudes: RL95-2, 9.3-fold; SPAC-1-L, re-expression and USPC-1, 36.5-fold (Fig. 2*b*). The outcomes were similar for DAC alone and combined TSA and DAC treatment in all cell-lines; however, TSA alone failed to produce either up-regulation or re-expression (Fig. 2*b*). Correlations of the status of miR-34b promoter methylation with p53 mutation, and miR-34b expression level in these cell lines are shown in Table 2.

Restoration of miR-34b inhibited growth, migration and invasion and promoted apoptosis activity of ESC cells

To determine whether miR-34b suppressed cancer cell growth, SPAC-1-L and USPC-1 cells were transfected with miR-34b precursor molecules or a negative control. Statistically significant reductions in proliferation by 8.3% ($p = 0.003$) and 16.1% ($p = 0.002$) in comparison to the control were observed on miR-34b transfection of SPAC-1-L and USPC-1 cells, respectively (Fig. 3*a*). As this may have been the result of apoptosis, we measured caspase activity. As shown in Figure 3*b*, overexpression of miR-34b precursor molecules led to a statistically significant increase in caspase 3/7 activity (NC 247.8 ± 6.8 *vs.* miR-34b 284.4 ± 17.7, $p = 0.03$ in SPAC-1-L and NC 4783.0 ± 339.4 *vs.* miR-34b 9589.7 ± 748.5, $p = 0.001$in USPC-1). miR-34b precursor molecules also produced caspase 3/7 increases in SPAC-1-L

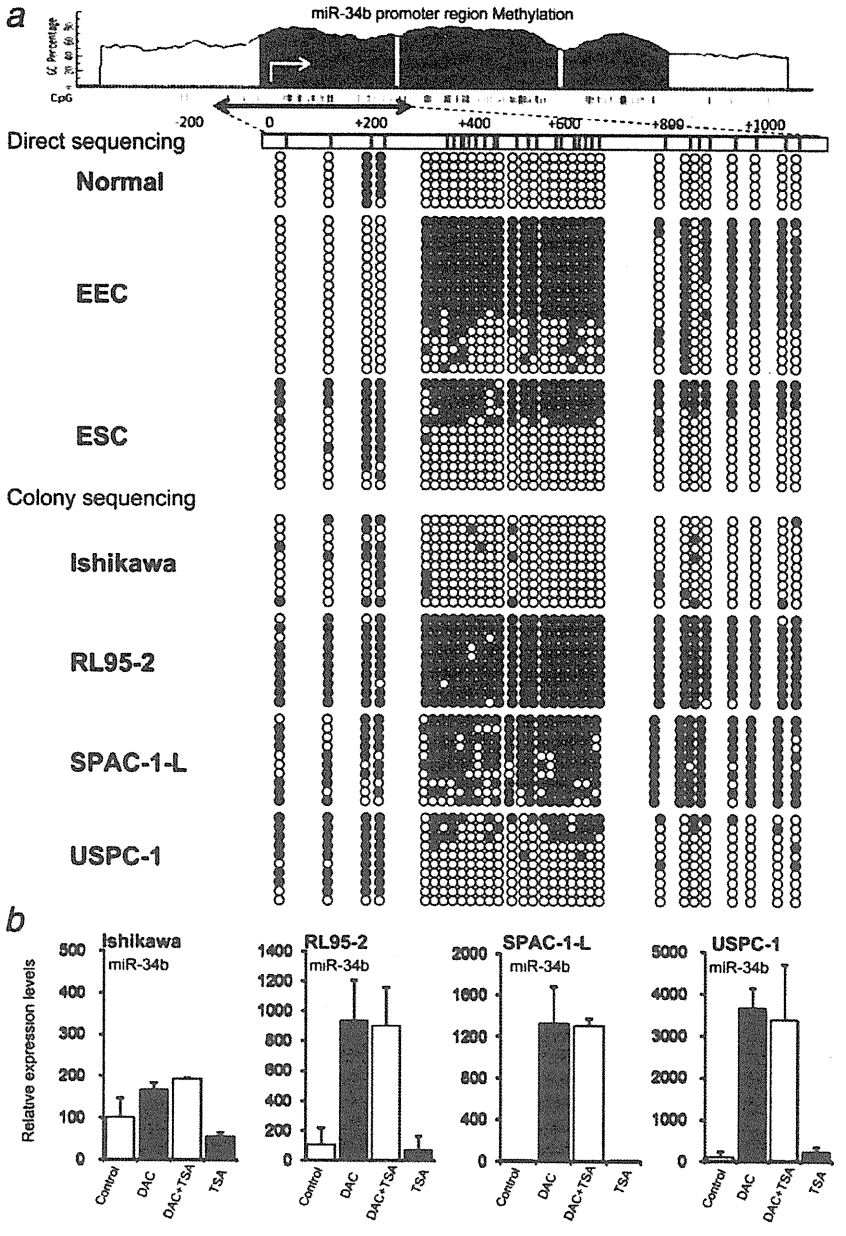

Figure 2. (*a*) Methylation status of the miR-34b promoter region determined by bisulfite sequencing in primary endometrial adenocarcinoma specimens and normal endometrial tissues, and bisulfite sequencing of clonal populations of endometrial cancer cell lines. In cell lines, 10 single clones are represented for each sample. Methylation status of 31 CpG sites detected by bisulfite sequencing is displayed. Solid circles (●) indicate the methylated CpG sites; open circles (○), unmethylated sites. Normal, normal endometrial tissues. (*b*) Quantitative RT-PCR results for miR-34b in the indicated endometrial cancer cell lines, with and without DAC and/or TSA treatment. Results are normalized to internal U6 snRNA expression. Columns, means of three replicates; error bars, standard deviations. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and USPC-1 cells in comparison to the precursor-transfected negative control, as assessed by flow cytometry.

In a migration assay, a 42% decrease in SPAC-1-L ($p = 0.001$) and 16.6% decrease in USPC-1 ($p = 0.006$) were observed on transfection of the miR-34b precursor (Fig. 3*d*). In the invasion assay, an 80% decrease in SPAC-1-L ($p < 0.0001$) and 78.7% decrease in USPC-1 ($p = 0.0004$) were observed (Fig. 3*e*). The most striking decrease was observed in invasion assays after miR-34b precursor molecules were transfected into SPAC-1-L cells.

Table 2. The status of methylation of miR-34b promoter, p53 mutation and miR-34b expression level in the cell lines

Cell line	Methylation of miR-34b promoter (%)	miR-34b relative expression level	Status of p53 mutation	Exon	Protein change
Ishikawa	10	525.7	(+) p	7	M246X
RL95-2	96	3.3	(+) d	6	V218-
SPAC-1-L	76	1	(+) p	8	P273X
USPC-1	25	10.1	(+) p	7	R248W

p, point mutation; d, deletion.

Correlation between miR-34b expression and MET protein expression in endometrial cancer cell lines and primary ESC

To evaluate if miR-34b modulated the level of MET in SPAC-1-L and USPC-1 cells, MET expression was measured by Western blotting after transfection with miR-34b precursor molecules. As shown in Figure 4*a*, 72 hr after miR-34b transfection, the amount of MET protein was significantly reduced in both cell lines.

MET immunoreactivity was detected in both the membrane and the cytoplasm of cancer cells (Fig. 4*b*). Greater reactivity was noted in the infiltrating cells at the tumor periphery. Patients with ESC were subdivided into three subgroups: negative/lower positive; negative and strongly positive and positive.[28] Fourteen out of 21 (66.7%) ESC cases were positive for MET immunoreactivity (Supporting Information, Table S1). In contrast, all normal cases were negative. Lower expression of miR-34b was significantly correlated with MET overexpression ($p = 0.012$; Fig. 4*c*).

Discussion

Our study demonstrated that miR-34b expression was significantly down-regulated in ESC tissues compared with normal endometrial tissues. In addition, there was a significant correlation between negatively regulated miR-34b expression and the rate of positive MET immunostaining. Negatively regulated miR-34b was associated with CpG island hypermethylation, as demethylation by DAC restored the expression of miR-34b. Restoration of miR-34b induced apoptosis and inhibited cell growth, migration and most notably invasion. In addition, MET protein expression was reduced by the restoration of miR-34b expression. To our knowledge, our report is the first one examining the effect of ectopic miR-34b expression on cancer cell behavior in ESC.

ESC is characterized by a high rate of p53 mutation.[4] p53 directly inhibits the expression of miR-34b/c and miR-34a in addition to its classical effect of inducing p21 expression and arresting the cell cycle.[13–18] miR-34a is encoded by a unique transcript, whereas mi-34b and miR-34c share a common primary transcript.[31] In ESC cases, our miRNA microarray analysis revealed that miR-34b was the second most down-regulated gene (40.2-fold compared with the normal endometrial tissue) and that miR-34c-5p was the 33rd most down-regulated gene (6.2-fold). The reverse strand of miR-34b* was also decreased 3.0-fold.[32] The other three miRNAs exhibited concommitant down-regulation, suggesting that they were polycistronic. In addition, miR-34b showed the greatest degree of specific down-regulation in ESC tissues compared with normal endometria. The alterations in gene expression were paralleled by the immunohistochemical data. miR-34b expression was low in ESC, in which immunostaining was strongly positive for p53, and higher in tumor samples without abnormal p53 accumulation. It is plausible that the down-regulation of miR-34b expression results from p53 dysfunction, as miR-34b is the target of p53.[13–18]

Dysregulation of miRNA expression occurs through several mechanisms including the gain or loss of gene copy number,[33] structural mutations (germline mutations) in miRNA precursor molecules,[34] promoter methylation,[35] abnormal miRNA processing[36] and transcriptional regulators.[13] Besides p53 mutation, epigenetic regulation has recently been identified in miR-34b. Specifically, hypermethylation of the miR-34b/c promoter region has been found in a number of tumor types.[19,37] In melanoma, a correlation has been shown between methylation status and metastatic potential.[20] In nonsmall cell lung cancer, however, Boomer *et al.* demonstrated that loss of miR-34b expression was correlated with p53 deletion and not with CpG methylation.[15] Thus, multiple mechanisms are thought to exist for the regulation of miR-34b expression. In our study, miR-34b promoter hypermethylation was observed in both EEC and ESC and at a lower frequency in ESC. In both EEC- and ESC-derived cell lines, the use of a demethylating agent restored miR-34b expression which had been low due to hypermethylation indicating that methylation was likely responsible for down-regulation of miR-34b in both lines. Further studies are needed to ascertain whether both p53 mutation and promoter methylation are contributing to the aberrant miR-34b expression found in ESC.

miR-34b/c modulates cell proliferation and regulates the cell cycle in a manner consistent with a tumor suppressor. Ectopic miR-34b/c expression in IM90 fibroblast cells decreases growth and induces senescence.[14] Additionally, in a variety of cancer cells, ectopic miR-34b/c expression induces G1 cell-cycle arrest.[14,15,18] Anchorage independent growth is also inhibited by miR-34b/c.[13] Our results were consistent in that restoration of miR-34b slightly but significantly reduced cell growth, suggesting an indirect effect.

In our study, apoptosis may have contributed to the decrease in cell number following ectopic expression of miR-34b/c. This is similar to what has been observed in nonsmall lung cancer cells in which miR-34a presumably interacts with other apoptotic pathway genes.[16] Similarly, our results indicated that miR-34b cooperated with other factors to promote apoptosis in ESC. However, the increase of apoptosis was moderate, which suggested that this phenotype was an indirect effect.

A clinically significant finding was that miR-34b in the ESC cells significantly inhibited migration and invasion. Our results showed that the inhibitory effects of miR-34b were more

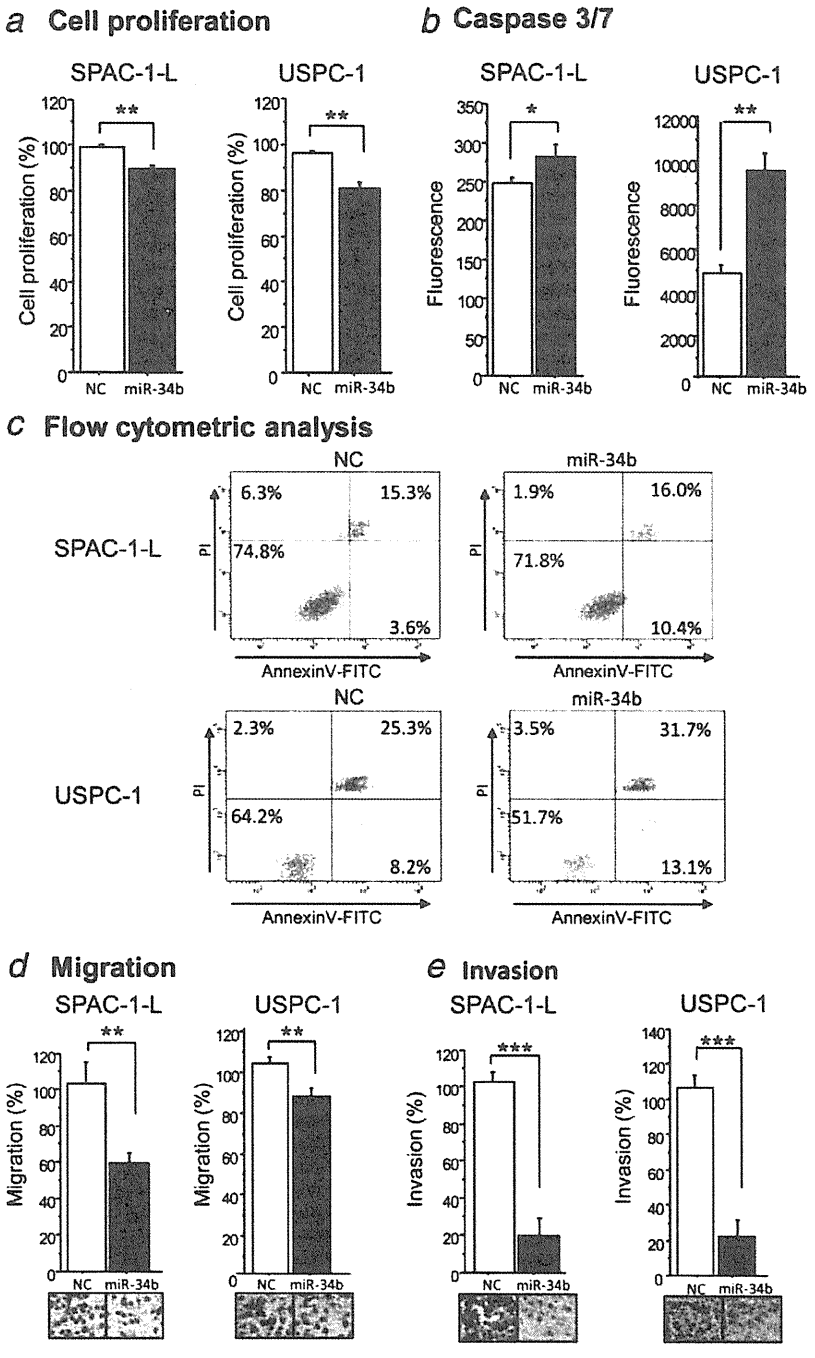

Figure 3. SPAC-1-L cells and USPC-1 cells were transfected with pre-miR-34b precursor molecules, or a negative control for 72 hr. (*a*) Cell proliferation assay; (*b*) Caspase 3/7 Glo assay. NC, transfected with negative control; 34b, transfected with pre-miR-34b molecules. Columns, means of three replications; error bars, standard deviations. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ *versus* controls. (*c*) Flow cytometric analysis. Cell death was monitored by Annexin V staining and flow cytometry. The right lower quadrant of each plot contains early apoptotic cells, whereas the right upper quadrant contains late apoptotic cells. This experiment was repeated three independent times and similar results were obtained each time. PI, propidium iodide. (*d*) Migration assay; (*e*) invasion assay. NC, transfected with negative control; miR-34b, transfected with pre-miR-34b molecules. Columns, means of three replications; error bars, standard deviations. $**p < 0.01$ and $***p < 0.001$ *versus* controls. Fields shown in the figure are representative of three replications. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

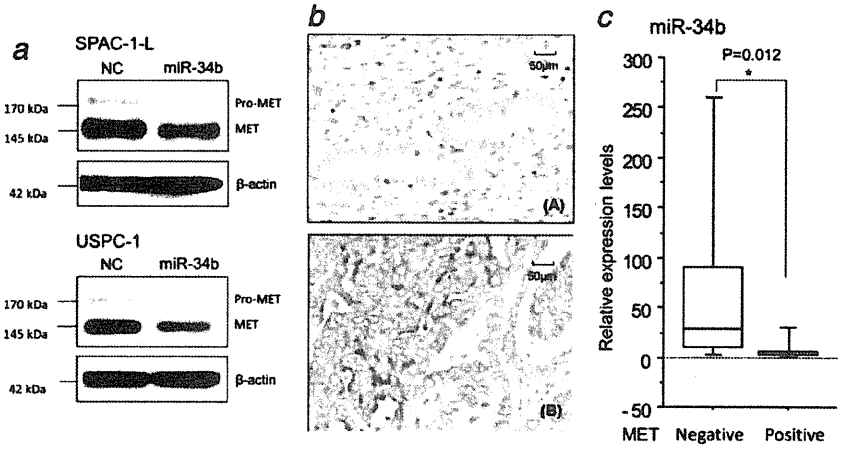

Figure 4. (*a*) Immunoblotting for MET in SPAC-1-L and USPC-1 cells. Specific bands corresponded to MET and β-actin immunoreactivity (approximately 145 and 42 kDa, respectively). MET antibody recognized both pro-MET and mature MET (approximately 175 and 145 kDa, respectively). MET immunoreactivity was clearly diminished in cells transfected with pre-miR-34b precursor molecules after 72 hr compared with negative control cells. NC, transfected with negative control; miR-34b, transfected with pre-miR-34b molecules. (*b*) Immunohistochemistry of MET expression in ESCs: (A) MET immunoreactivity was negative in all normal endometrial tissues and (B) MET immunoreactivity was detected in both the membrane and the cytoplasm of cancer cells. Original magnification, 200× for (A) and (B). (*c*) miR-34b expression was significantly lower in tissues exhibiting positive MET immunoreactivity. Negative, MET negative and weakly positive immunoreactivity; Positive, MET strongly positive immunoreactivity. Columns, means of three replicates; error bars, standard deviations. $*p < 0.05$.

pronounced in ESC than in EEC. It is plausible that these observations are the result of alterations in the expression of genes that are downstream targets of miR-34b.

MET, c-MYC, CDK6 and CREB are putative target genes of miR-34b.[38] Of these, MET, through activation, is involved in several critical steps in tumor development and metastatic dissemination.[22] MET is a direct target of the miR-34 family in ovarian cancer cells.[23,39] miR-34b/c binds to the 3′UTR of MET and impairs MET mRNA translation.[19,23,39] Furthermore, the migratory and invasive activities of the cancer cells are controlled by the negative regulation of MET *via* miR-34b/c.[23,39]

MET expression is a significant prognostic factor in EEC patients.[40] Consistent with these results, we obtained markedly reduced levels of MET protein by transfection of miR-34b precursor molecules. This suggests that MET is in part responsible for the invasive behavior of ESC cell lines. Additionally, MET overexpression correlated with miR-34b reduction in primary cancer specimens. These results suggest that miR-34b might function as a tumor suppressor by regulating invasive growth *via* MET. To our knowledge, this report is the first to show the correlation between miRNA-mediated MET expression and invasion in ESC cells.

Our findings, taken together with the previously discussed studies, suggest that miRNAs such as miR-34b/c have a tumor suppressive function. Therefore, this pathway may represent a potential therapeutic target. Preliminary studies have already been completed in xenograft models. For example, the administration of a miR-34a/atelocollagen complex has been shown to inhibit tumor growth of colon cancer cell lines in xenograft models.[41] The reintroduction of miR-34b/c has been reported to inhibit the migratory ability, tumor growth and metastasis formation in head and neck cancer lines in xenograft models.[20] Further studies are needed to determine whether restoration of miR-34 will have a therapeutic role in ESC.

In conclusion, we have shown that miR-34b is down-regulated in endometrial carcinomas. The inhibition of cell growth and increase in apoptosis were confirmed by transfection of the miR-34b. Although the effect was statistically significant, the overall magnitude was weak. Therefore, the actions of miR-34b are likely indirect. miR-34b down-regulation is also associated with increased migration and invasion, suggesting that this pathway is involved in the aggressive behavior of ESC. Further studies are needed to verify whether this pathway represents a viable therapeutic target.

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